Determination of Hepatitis C Viral Load and Genotypes by Real-Time and RT-PCR at Thi_Qar Province

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الخلاصة

أجريت هذه الدراسة للفترة من أيلول 2010 إلى تموز2011 ، للكشف عن الحمل الفيروسي وتحديد الأنماط الجينية لفيروس التهاب الكبد ج بين الأشخاص الذين لا يظهرون الأعراض والمرضى من المراجعين الى مركز الدم الرئيس ، مركز الأمراض الوراثية /فقر دم البحر المتوسط /مستشفى الحبوبي ، وحدة غسيل الكلى/مستشفى الحسين التعليمي ومختبر الصحة العامة في محافظة ذي قار.اتبعت تقنية تفاعل سلسلة التضاعف المعاكس الكمي ذو الوقت الحقيقي لاختبار (90) عينة مصل من أفراد اظهروا كشوفا مصلية موجبة لأضداد فيروس التهاب الكبد ج بواسطة تقنية المعايرة الامتصاصية المرتبطة بالأنزيم وأكدت اصابة 51(56.66%)منهم، وبينت هذه التقنية أنّ الأحمالَ الفيروسية في الدم تراوحت بين 1.19 ×10^3 إلى 4.3 × 10^6 وحدة عالمية/مل مِنْ الدمِّ وكان المتوسطِ الحسابي للحملِ الفيروسي 5.9 × 10^5 وحدة عالمية/مل، فيما كان الوسيط 2.6 × 10^5 وحدة عالمية/مل. حددت ثلاثة أنماط جينية لفيروس التهاب الكبد نمط ج (1أ، 1ب و 4) وكان النمط الجيني 4 هو السائد وبنسبة (86.27%) ، تلاه (1ب) (37.25%) و (1أ) (33.3%) وبفارق معنوي. وجد النمط الوراثي 4 في 44 من أصل 51 حالة منها 21 (41.18%) كانت إصابات مفردة. الإصابات المختلطة بالنمط الوراثي 4 مع كل من (1أ) و (1ب) على انفراد وجدت في 21.57% و 11.76% على التوالي. الإصابات المختلطة بالنمط 4 والنمطين (1أ) و (1ب) مجتمعة وجدت في 11.76%. فيما كانت نسبة الإصابات المنفردة بالنمط (1ب) هي 13.73% في حين لم تسجل إصابات منفردة بالنمط الوراثي (1أ). تعد هذه الدراسة الأولى من نوعها في محافظة ذي قار والتي تضمنت الكشف عن الأنماط الجينية وتحديد الحمل الفيروسي لفيروس التهاب الكبد ج باستخدام تقنية تفاعل سلسلة التضاعف المعاكس و تقنية تفاعل سلسلة التضاعف المعاكس الكمي ذو الوقت الحقيقي.

Abstract

The present study was carried out from Sept. 2010 to Jul. 2011 to detect the viral load and genotypes of HCV infections among asymptomatic peoples and patients referred to the central blood bank, center of thalassemia /AL-Haboby Hospital, renal dialysis unit/Al-Hussein Teaching Hospital and public health laboratory at Thi-Qar province.

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Real-Time Polymerase Chain Reaction Technique (RT-qPCR) was implemented on 90 individuals of anti-HCV seropositive by ELISA III assay. The results revealed that 51(56.66%) were gave positive results for HCV. The RT-qPCR analyses of the positive samples were showed that the viral loads were ranged from 1.19×10^3 to 4.3×10^6 IU/ml of blood .The mean of viral load was 5.9×10^5 IU/ml of blood, whilst, the median was 2.6×10^5 IU/ml. Three genotypes of HCV were detected in patients serum by RT-PCR technique. Genotype 1a (33.33%), genotype 1b (37.25%) and genotype 4 (86.27%) with significant differences ($p>0.05$). Genotype 4 was the predominant and found in 44 of 51 cases, of those 21(41.18%) as single infections. Mixed infections with genotype 4 and each of 1a and 1b was found in 21.57% and 11.76% respectively. Mixed infections with genotype 4 and both 1a and 1b was found in 11.76%. Single infections with genotype 1b only was found in 13.73%, while genotype 1a was detected only in mixed infections. This study was the first at Thi-Qar province which involved the searching for HCV viral load and genotypes by using of Real-Time and RT-PCR technique.

Introduction

Chronic hepatitis C infection is now recognized as an important health problems, as a major cause of chronic liver disease world wide[1,2,3]. Particularly cirrhosis and hepatocellular carcinoma[4,5]. Infection is transmitted primarily by exposure to blood or blood products that have not undergone screening or viral inactivation, or by shared needles and syringes during unsafe medical procedures[6]. WHO estimated that approximately 170 million people are infected with HCV world wide [7].

HCV discovered in 1989 as non-A , non-B hepatitis[8,9], and classified in *Hepacivirus* genus of the Flaviviridae family, and its genome is a positive-stranded RNA of 9.6 kb in length[10], and it encodes a core protein, two enveloped glycoproteins, and several non structural proteins [11]. The genome of HCV is highly variable. So far, six major genotype and more than one hundred subtype have been described[12]. The error-prone RNA polymerase of HCV

together with the high replication rate of the virus is responsible for the large intrpatient genetic diversity of HCV strains[13].

The viral load is the amount of specific viruses found in a given volume of blood from infected patient. More precisely, it means the amount of HCV genetic materials that found in the blood corresponds to as many hepatitis C viral particles as the given number says, therefore the given number denotes as viral equivalents, also termed as viral copies or viral titer[15]. Using the WHO international standard, a VL (Viral Load) of 2 millions copies/ml (the cut-off value predictive for therapeutic success in early clinical trials with IFN) was found to correspond to 800 000 IU/ml[16]. Methods for accurate quantitative of serum and plasma hepatitis C virus (HCV) RNA levels have become key tools both for understanding the biology of HCV infection and for the clinical management of patients under treatment. The ability to predict likelihood of response to combination interferon/ribavirin therapy by assessing rates of HCV viral load decline has provided a more individualized treatment algorithm that can identify non-responsive patients early in treatment, sparing them significant morbidity and cost[14].

HCV RNA should be tested with highly sensitive assays after 24 weeks of treatment because patients with detectable HCV RNA at this time point only have a 1-2% chance of achieving SVR. SVR, defined as the absence of detectable HCV RNA 24 weeks after treatment completion, should be assessed by an HCV RNA detection assay with a lower limit of 50 IU/ml or less to evaluate long-lasting treatment success [17].

Many studies have indicated an association between HCV genotype and responsiveness to alpha interferon treatment[18,19]. It is now well documented that therapeutic outcome of antiviral treatment is influenced by the virus genotype. A prior knowledge of the genotype before therapy has become an important aspect of therapeutic strategy, because of its predictive value in terms of the response to antiviral therapy. Differences in geographic distribution of HCV genotype have also observed [20,21,22].The present study was

aimed to detected viral load and genotypes prevalence among patients at Thi-Qar province.

Materials and Methods

Patients: This study was carried out from Sept. 2010 to Jul. 2011 on asymptomatic peoples and patients referred to the center of thalassemia /AL-Haboby Hospital, central blood bank, renal dialysis unit/Al-Hussein Teaching Hospital and public health laboratory at Thi-Qar province.A total (90) serum samples(sero-positive for HCV by ELISA III assay) were collected from patients.

HCV RNA and viral load detection: AccuPerp viral RNA extraction kit (Bioneer, Korea) was used for RNA extraction from serum samples. Five µl of viral RNA was used for Real-Time PCR amplification steps by using AccuPower® HCV Quantitative Real-Time PCR kit (Bioneer, Korea). The Real Time PCR Thermocycler : Exicycler™ Quantitative Themal Block, Version 3.0, was loaded , and run by the following program (Wittwer *et.al.,* 1997) [23] :

Steps	Temperature	Running Time
Line 1 : Reverse Transcription	45 C°	15 minutes
Line 2 : Pre-Denaturation	95 C°	5 minutes
Line 3 : Denaturation	95 C°	5 seconds
Line 4 : Annealing & Extension	55 C°	5 seconds
scan	Fam-BHQ : HCV / Tamra-BHQ: Internal Positive control	
Go to	Line 3, 45 cycles	

Once the program began, the graphics that indicate progressing of multiplexing have been appeared. The result appeared on the screen after the program was done. The standard curve was constructed after addition (1µl) of serial HCV standard RNA from $2\times 10^2 - 2 \times 10^7$ copies/µl to six wells of RT-qPCR PreMix, then 44µl from PCR Grad water was added and (1µl) of internal positive control RNA. After doing the mentioned procedure, six C_t values (C_t : fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background values) were

resulted and the standard curve was automatically plotted through the obtained Ct values against logarithms of transformed concentrations of serial standard RNA as figure (1) :

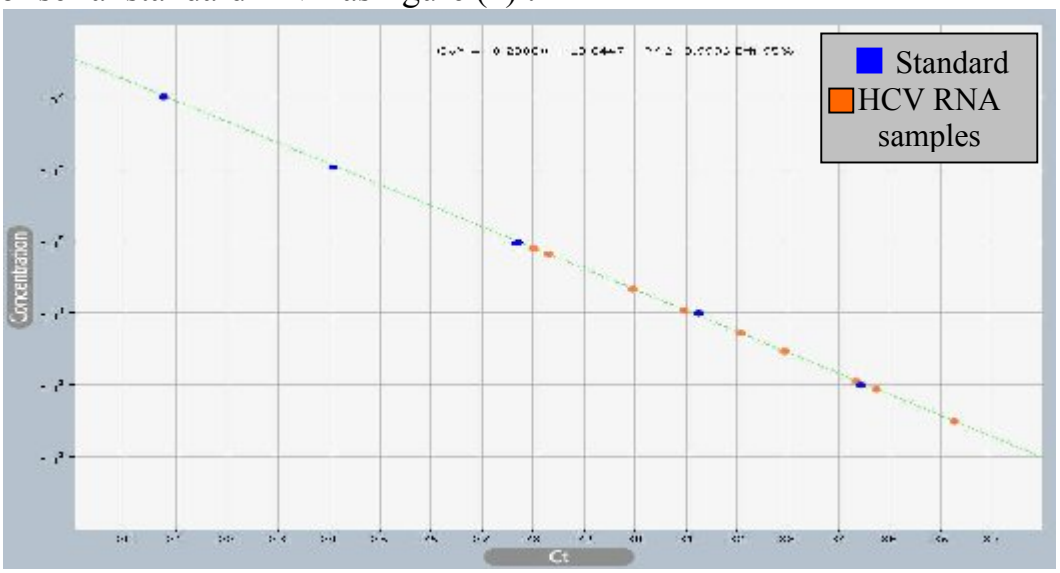


Figure (1) : Standard curve obtained by Excicycler Real-Time PCR.

Genotyping: serum samples which showed HCV RNA positive Real-Time PCR technique, were examined by RT-PCR to detect genotypes. Five µl of viral RNA(extracted as in previous step) was used for cDNA synthesis and amplification steps by using AccuPower® RT-PCR PreMix kit (Bioneer, Korea), which contains all the component necessary for cDNA synthesis and amplification in one tube. Mixture of primers set used according to Ohno *et.al*., [24] for RT-PCR are listed in table (1):

Table (1) : Primers set for HCV genotype.

Primer	Sequence (5´-3´)	Nucleotide position
Sc2	CGGAGGTCTCGTAGACCGTGCACCATG	224-3
Ac2	GAGACGGGTATAGTACCCCATGAGAGTCGGC	417-391
S7	AGACCGTGCACCATGAGCAC	212-8
A5	TACGCCGGGGGGTCATGTGAGGGCCCCA	343-319
	Mix.1	
S7	AGACCGTGCACCATGAGCAC	212-8
S2a	AACACTAACCGTCGCCCACAA	60-40
G1b	CCTGCCCTCGGGTTGGCTAAG	222-203
G2a	CACGTGGCTGGGATCGCTCC	178-159
G2b	GGCCCCAATTAGGACGAGAC	325-306
G3b	CGCTCGGAAGTCTTACGTAC	164-145
	Mix.2	
S7	AGACCGTGCACCATGAGCAC	212-8
G1a	GGATAGGCTGACGTCTACCT	196-177
G3a	GCCCAGGACCGGCCTTCGCT	220-211
G4	CCCGGGAACTTAACGTCCAT	87-68
G5a	GAACCTCGGGGGCAGAGCAA	308-289
G6a	GGTCATTGGGGCCCCCAATGT	334-315

The PCR amplification condition were achieved according to Ohno *et.al.*[24] for 40 cycles as first round and 30 cycles of amplification as second round as follows:

A-First round : One µl from each primer solution Sc2, Ac2, S7 and A5 were added to cDNA ´s product tube. Nuclease free water was added to a final volume 50µl. Thermocycler was loaded as the following:

20 preliminary cycles :

Steps	Temperature	Time
1	94°C	1 minute
2	45°C	1 minute
3	72°C	1 minute

20 other cycles :

Steps	Temperature	Time
1	94°C	1 minute
2	60°C	1 minute
3	72°C	1 minute

B- Second round :

It was done immediately after first round. (25)µl from the PCR products of the first round were putted into separated tube A and B .

One µl of each primer from mixture 1 was added for A tubes and 1µl of each primer from mixture 2 for B tubes. Nuclease free water was added to each tube to final volume 50µl. Thermocycler was loaded as the following :

30 cycles :

Steps	Temperature	Time
1	94°C	1 minute
2	62°C	45 seconds
3	72°C	1 minute

Eight microliters of the second round PCR product was electrophoresed on a 2% agarose gel, stained with ethidium bromide, along with 100-3000 bp DNA as size marker and visualized under UV-transilluminator. Genotype specific bands were marked according to their molecule weight in comparison with DNA ladder 1a (208 bp) , 1b (234 bp) and 4 (99 bp) (Ohno *et.al*.,)[32].

Results

The study was conducted on 90 of anti-HCV sero-positive individuals of both sexes gathered comprising as: (42) blood donors; (34) thalassemic patients; (9) polycythemic patients; (3) from medical staff and (2) renal dialysis patients. The molecular quantitative technique with Real-Time PCR was used to confirmed the serological diagnosis and for measurement of the viral loads (concentrations) in the blood of HCV patients .The results revealed that 51(56.66%) of seropositive patients were gave positive results for HCV RNA, 22(43.14%) blood donors; 20(39.22%); thalassemic patients; 6(11.76%)polycythemic patients; 2(3.92%) medical staff and 1(1.96%) renal dialysis patients.

viral loads were ranged from 1.19 × 103 IU/ ml to 4.3 × 106 IU/ml of blood. The mean of viral load was (5.9 × 105) IU/ml , whilst, the median was (2.6 × 105) IU/ml of the blood.

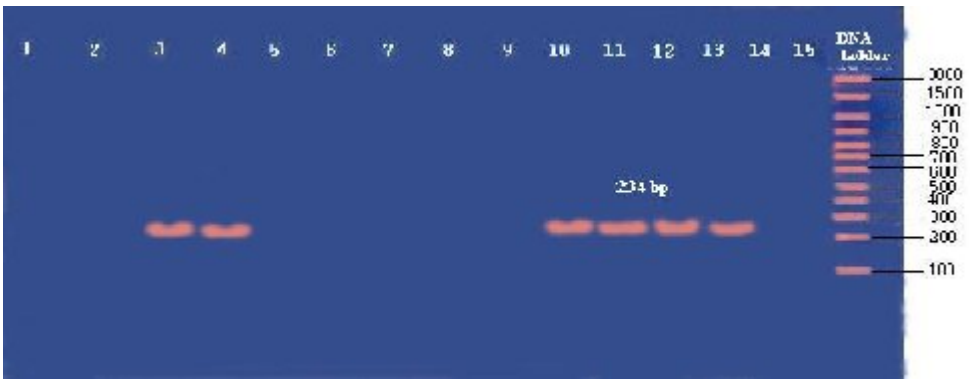
The results of RT-PCR showed that three genotypes of HCV were detected in patients serum, genotype 1a (33.33%), genotype 1b (37.25%) and genotype 4 (86.27%) with significant differences ($p>0.05$). Genotype 4 was the predominant and found in 44 of 51 cases (86.27%), of those 21(41.18%) as single infections. Mixed

infections with genotype 4 and each of 1a and 1b was found in 21.57% and 11.76% respectively. Mixed infections with genotype 4 and both 1a and 1b was found in 11.76%. Single infections with genotype 1b only was found in 13.73%, while genotype 1a was detected only in mixed infections (Table 2 ; Fig. 1).

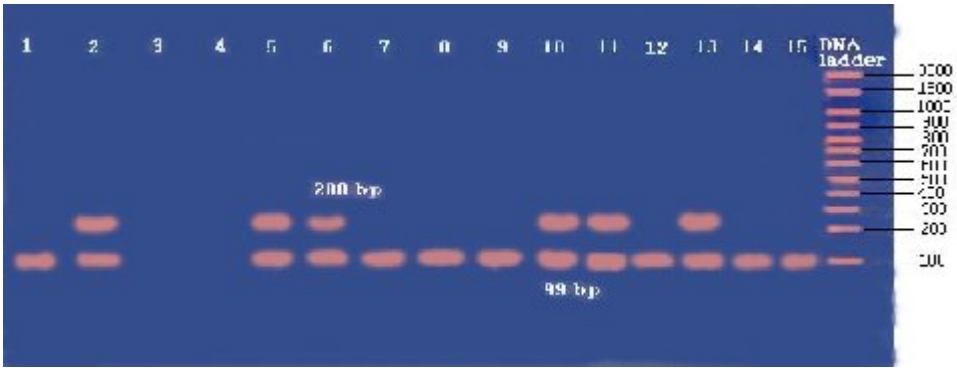
Table(2): Genotyping of HCV by using RT-PCR analysis in the study groups.

Study group Genotype	Blood donors	Thalassemic patients	Renal dialysis	Medical staff	Polycythae-mia patients	Total
1b	3	2	0	0	2	7(13.73%)
4	13	3	0	2	3	21(41.18%
Mixed (1a+4)	2	8	1	0	0	11(21.57%)
Mixed (1b+4)	4	1	0	0	1	6(11.76%)
Mixed (1a+1b+4)	0	6	0	0	0	6(11.76%)

Mixture 1



Mixture 2



(Fig. 2): Bands of HCV genotypes obtained by RT-PCR using two mixture of primers.

* Mixture 1 : Lanes 3, 4, 10, 11, 12 and 13	Genotype 1b
* Mixture 2 : Lanes 1, 7, 8, 9, 12, 14 and 15	Genotype 4
Lanes 2, 5, 6, 10, 11 and 13	Mixed infection (1a + 4)

Discussion

The results of RTq-PCR revealed that HCV-RNA positive was detected in 51(56.66%) of (90) of anti-HCV sero-positive. The reasons where the discrepancy between the antibody positive and HCV-RNA negative cases existed, might be that HCV may be present in peripheral blood mononuclear cells (PBMCs) in those cases and not in serum or plasma as has been reported by Caudai *et.al.,*[25] who detected HCV-RNA in PBMCs in 10.5% out of 38 plasma viremia negative, and that the spontaneous viral clearance was occur in twenty percent of individual exposed to the virus[26], therefore the presence of anti-IgG reflect resolved infection. Current antiviral therapy is based on the combination of a interferon with ribavirin resulting in an overall sustained virological response (SVR) of about 60% [27]. About 6% of total cases in present study were under treatment. This also may belong to the specificity of serological test. Carey,[28] mentioned that ELISA-III is the most accurate serological marker for diagnosis of HCV infections but it still gave false positive and false negative results and cannot discriminate between the past and ongoing infections.

The viral loads in blood of HCV patients were ranged from 1.19 × 10^3 IU/ ml to 4.3 × 10^6 IU/ml and the mean was 5.9×10^5 IU/ml , whilst, the median was 2.6×10^5 IU/ml of the blood. This wide range in the viral blood titer may be set due to the variety of the patients whom were from various groups, ages and the stages of the disease. The immune response and the current status of the patient were also have important role in the fluctuation of the viral load at the same individual[29,30]. This results were less than results obtained by Abdul-Sada**,** (2011)[31],were the viral loads ranged from 10^2 to 4.5 × 10^8 IU/ml of blood, whereas, the mean and median were 5.8 × 10^6 and 3.6 × 10^4 IU /ml of blood respectively. The correlation between HCV genotype and viral load remains controversial. In some studies high titer viraemia was correlated with advanced liver stage[32], while others found correlation with HCV genotype[33].The present study was showed no fluctuation in viral load among patients groups because about 86.27% of infections belonged to the same genotype (HCV-4).

HCV genotyping may shed light on its evolution, source of outbreaks, and risk factors. It may be used to identify the source of infection in cases of patient-to-patient transmission and is also useful in the study of other modes such as vertical (mother to baby), sexual transmission and needle stick injury [34].

By using RT-PCR, three HCV genotype (1a, 1b and 4) were identified, and genotype 4 was the predominant (86.27%), followed by 1b (37.25%), and 1a (33.33%) each of which had a specific epidemiological profile. This study was the first at Thi-Qar province which involved the searching of HCV genotypes among asymptomatic patients by using of RT-PCR test.

The present study is in agreement with two other studies, first by Al-Kubaisy *et.al.*,[35] which have studied the seroprevalence of HCV genotypes by use of genotype specific ELISA-III test searching antibodies at Iraqi thalassemic children, they were reported that genotype 4 was the most frequent type followed by 1a and 1b, and showed mixed infection with genotypes 1a and 4. The other study by Abdul-Sada**,**[31], revealed that the genotype 4 is the predominant

and found in 89.4% of the infected cases followed by genotypes 1b, 2b, 3a and 6a with a percentages of 6.79%, 2.91%, 2.91% and 1.94% respectively. These studies including the present are confirmed the prevalence of genotype 4 in Iraq.

The epidemiological profile of genotype 4 consist of immigrants from other countries, mainly Egyptian immigrants were infected with HCV-4 in their home country. Egypt has the highest prevalence of HCV infection in the world due to the use of unsterile equipment during mass treatment of the population with parenteral antischistosomal therapy from 1920s to 1980s[36]. Approximately 90% of Egyptian isolates belong to HCV-4[37], An unnoticed nosocomial HCV-4 outbreak before 1990 may be another possible explanation for the high prevalence of genotype 4 observed in this study.

The epidemiological profile of genotype 1a was linked to IDU, and the genotype 1b is more often with patients who acquired HCV through blood transfusion (38).

Persons can be infected with several genotypes or subgenotypes of HCV following exposure to blood or blood products. Possible explanations for mixed infections include initial exposure to multiple viruses, such as occurred from receipt of clotting-factor concentrates prepared from multiple donors prior to HCV testing and inactivation, or from exposure to multiple viruses over time among networks of injection drug users(39), this explain the mixing infections by (1a, 1b and 4) which observed in present study.

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